

MICROBIAL OXIDATION AND UTILIZATION OF ORTHOPHOSPHITE DURING GROWTH¹

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In the soil there exist cycles of biological oxidation and reduction for inorganic compounds of sulfur and nitrogen. We have been interested in the possibility that a similar cycle might exist for inorganic compounds of phosphorus. In particular, we have been interested in one step of such a cycle, that of biological oxidation of orthophosphite to orthophosphate.

There are few reports in the literature pertaining to the utilization of phosphite phosphorus by microorganisms. Dox (1911), in reporting studies on the growth of *Aspergillus niger* on media containing various phosphorus sources, concluded that phosphite phosphorus was neither available nor toxic to the fungus. No growth occurred when phosphite was the only phosphorus source but growth did occur if phosphate also was present in the medium.

Robertson and Boyer (1956a, b) considered orthophosphite and various organic phosphite compounds to be inert in biological systems. In fact, these workers proposed that orthophosphite be used as a buffer for biological studies in the range of pH 5.5 to 7.5. A later report (Bulen and Frear, 1957) has shown, however, that for some biological systems phosphite cannot be used as a buffer in this pH range. In this instance it was found that phosphite inhibited growth of *Azotobacter agilis* strain O (*A. vinelandii*) when cultured in Burk's medium with gaseous nitrogen as the nitrogen source. Inhibition was not observed when ammonium acetate served a similar function. Furthermore, these workers found that phosphite inhibited oxygen consumption by washed cells fixing nitrogen and that under these conditions the nitrogen fixation also was inhibited. Orthophosphate had little effect on these reactions.

The most convincing report of microbial oxidation of orthophosphite to orthophosphate

is that of Adams and Conrad (1953) who found that when a glucose-ammonia-phosphite medium was inoculated with a soil-solution culture of bacteria there was considerable loss of phosphite phosphorus from the medium during growth. However, the oxidation product, orthophosphate, did not accumulate in the medium although some of the phosphorus could be recovered as orthophosphate if the bacterial cells were caused to autolyze. Further experiments with pure cultures demonstrated that 5 out of 7 bacterial, 4 out of 4 fungal, and 3 out of 4 actinomycete strains could utilize phosphite phosphorus for growth although in no instance did orthophosphate accumulate in the culture broths.

Adams and Conrad also examined washed cells of 4 bacterial strains, in the presence and absence of a complete growth medium, for utilization of phosphite. In the absence of growth medium phosphite was not utilized but with the medium present phosphite disappeared from solution. Again, orthophosphate did not accumulate outside the cells. From these results the authors concluded that, "... the phosphite is absorbed or assimilated by actively growing microorganisms before it is oxidized."

The object of the present study was to find a microorganism(s) which could utilize phosphite phosphorus for growth and also would accumulate the oxidation product, orthophosphate, in the medium. It was felt that an organism of this type might allow some insight into the mechanism by which phosphite phosphorus becomes available to microorganisms for growth.

MATERIALS AND METHODS

Organisms and culture conditions. All cultures used in these studies were maintained on nutrient or potato-glucose agars (Difco) and are deposited in the culture collection of the Department of Bacteriology, The Pennsylvania State University. The author is indebted to C. C. Delwiche of the University of California at Berkeley for the

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strain of *Pseudomonas denitrificans*, and to P. W. Wilson of the University of Wisconsin for strains 9 and A-312 of *Pseudomonas fluorescens*. Strain C-II of *Pseudomonas aeruginosa* was received from B. Martineau of the University of Montreal.

Inoculum for all experiments was produced by transferring a loopful of slant growth to 50 ml of a liquid phosphite medium in a 250-ml Erlenmeyer flask. This medium, adjusted to pH 7 with KOH, contained per L: glucose, 0.5 g; yeast extract (Difco), 0.1 g; $(\text{NH}_4)_2\text{SO}_4$, 2 g; KCl, 0.1 g; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g; $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.01 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.03 g; CaCl_2 , trace; $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$, 2 g. The latter compound, as a 10 per cent solution, was adjusted to pH 7 with HCl and sterilized separately by passage through a sintered glass filter or by autoclaving 10 min at 121 C (15 lb pressure). The medium used for growth and phosphite oxidation studies differed from the above only in that the glucose concentration was at 2.5 g per L.

For growth and phosphite oxidation studies the growth medium was dispensed at 50 or 75 ml per 250-ml Erlenmeyer flask and was inoculated with 3 drops of a culture which had been shaken 48 hr at 25 C. These flasks then were shaken on a Brunswick rotary shaker at 25 C and samples were taken daily for analyses. In

those experiments conducted at 37 C a reciprocating shaker was used.

Analytical procedures. All samples (5 ml) taken for analyses were centrifuged to remove the cells. Orthophosphate phosphorus in the supernatant solutions was determined by the method of Dickman and Bray (1940). The procedure of Adams and Conrad (1953) for orthophosphite phosphorus was modified in that enough sulfuric acid was added to the samples to lower the pH below 6.

Residual carbohydrate in culture broths was determined by the anthrone method (Morris, 1948). Preliminary experiments indicated that $\text{Na}_2\text{HPO}_3 \cdot 5\text{H}_2\text{O}$ at the concentrations normally present in culture broths did not interfere in the determination.

Determinations of numbers of viable bacteria in culture broths were made by the plate count technique. The plating medium was nutrient agar (Difco) containing 0.1 per cent glucose.

RESULTS

Survey of microorganisms. A survey was made of pure cultures of microorganisms for their ability to utilize orthophosphite phosphorus for growth and to accumulate the oxidation product, orthophosphate, in culture broths. Nineteen cultures, which included bacteria, yeasts, acti-

TABLE 1
Growth of microbial cultures in phosphite medium

Microorganism	Strain	Relative Growth; 3 Days
<i>Pseudomonas aeruginosa</i>	C-II	+++
<i>Pseudomonas fluorescens</i>	P-195	+++
<i>Rhizobium meliloti</i>	102F29	—
<i>Rhizobium trifolii</i>	P-306	+++
<i>Rhizobium leguminosarum</i>	128C44	—
<i>Agrobacterium radiobacter</i>	ATCC-6466	+++
<i>Agrobacterium tumefaciens</i>	ATCC-11157	++
<i>Erwinia amylovora</i>	P-182	+++
<i>Escherichia coli</i>	P-167	+++
<i>Azotobacter chroococcum</i>	P-305	++
<i>Azotobacter agilis</i> (<i>A. vinelandii</i>).....	ATCC-9104	—
Soil isolate.....	7-25-6a	+++
<i>Staphylococcus aureus</i>	P-52	—
<i>Sarcina ureae</i>	P-67	—
<i>Bacillus subtilis</i>	P-11	—
<i>Saccharomyces cerevisiae</i>	P-257	+++
<i>Streptomyces griseus</i>	ATCC-3326	—
<i>Pythium debaryanum</i>	ATCC-9998	—
<i>Rhizoctonia solani</i>	ATCC-10154	—

nomycetes, and fungi, were tested and as may be seen in table 1, only 10 of these cultures grew during shaking for 3 days at 25 C. Nine out of the 10 were gram-negative bacteria. Analyses for orthophosphate in 0.5-ml samples of these cultures revealed that only *Pseudomonas fluorescens* strain 195 had accumulated free orthophosphate in the medium.

Other members of the genus *Pseudomonas*, therefore, were tested to see whether orthophosphate accumulation during growth on orthophosphite might be a general characteristic of this genus. Strains of *Pseudomonas aeruginosa* (P-197a and C-II), *P. denitrificans*, and *P. fluorescens* (P-195, 9, and A-312) were grown 4 days at both 25 C and 37 C and samples were taken daily for determination of orthophosphate phosphorus in the culture fluids. The two day samples also were plated for total numbers of viable organisms per ml.

The plate count determinations revealed that at 25 C all of the *Pseudomonas* strains grew well with phosphite phosphorus. Depending on the strain, the counts ranged from 0.7×10^6 to 4.8×10^6 cells per ml. Similar counts were obtained for growth at 37 C, except for *P. fluorescens* strains A-312 (5.4×10^5 cells per ml) and P-195, which did not grow at this temperature. During the rest of the incubation period the relative turbidities of the various cultures remained approximately the same.

In this experiment the culture broths of strain 195 grown at 25 C contained at the fourth day of sampling a total of 4.23 mg of orthophosphate phosphorus in 50 ml of broth. No orthophosphate phosphorus was detected in the supernatants of the other cultures regardless of whether grown at 25 C or 37 C. Based on these results *P. fluorescens* strain 195 was chosen for further study because it was felt that this organism allowed an examination of the conditions which might influence the ability of a microorganism to oxidize phosphite.

Fermentation analysis for growth of strain 195 on phosphite medium. A fermentation analysis was conducted to determine whether the accumulation of orthophosphate by strain 195 coincided with carbohydrate utilization and cell multiplication or whether it was the result of death and autolysis of old cells. Daily samples taken during 4 days of shaking at 25 C were centrifuged and the supernatant solutions analyzed for orthophosphate phosphorus, total

of orthophosphite plus orthophosphate phosphorus, pH, viable cells per ml and residual carbohydrate as glucose. It may be seen (figure 1) that although the most rapid rate of growth occurred within the first 24 hr, phosphate phosphorus did not begin to appear in the medium until the second day, from which time the concentration increased in a linear fashion through the third and sometimes the fourth day. Cessation of the linear appearance of phosphate phosphorus coincided approximately with the initiation of a maximal stationary phase of growth and with the disappearance of carbohydrate from the medium. The pH usually dropped to about 6 within the first 2 days, then rose slightly for the last two. The total phosphorus in the supernatant solutions, as the sum of phosphite and phosphate phosphorus, remained approximately the same during incubation and ranged from 21.0 to 22.6 mg.

Effect of medium constituent variation on phosphite oxidation. If phosphite oxidation is a function of cell growth and metabolism then variations in the medium constituents should have a marked effect on the rate of orthophosphate accumulation in culture broths. The effect

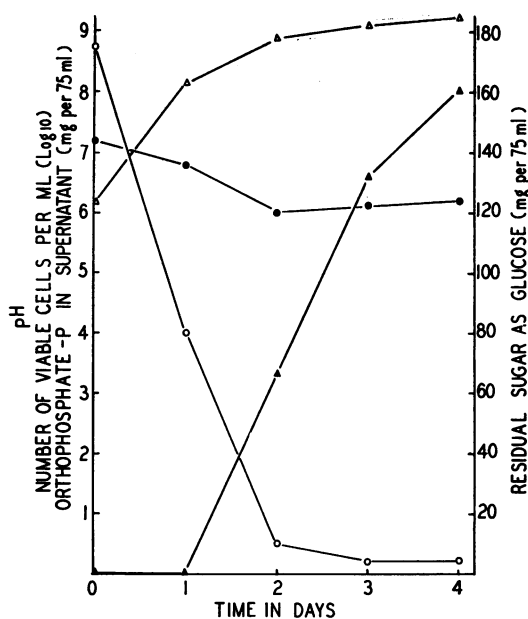


Figure 1. Fermentation analysis for *Pseudomonas fluorescens* strain 195 growing in a phosphite medium. Residual sugar, ○—○; pH, ●—●; number of viable cells, △—△; orthophosphate P, ▲—▲.

of the source of carbon for growth was studied by replacing the glucose of the medium with other carbon sources at the same concentration and with DL-alanine at 5 g per L. Alanine also was added at this concentration to a similar medium which did not contain ammonium sulfate. As may be seen from table 2, orthophosphate accumulation occurred only with glucose and alanine although by the fourth day there was a trace of phosphate in the supernatant solutions of cultures grown on acetate.

In a further experiment various constituents of the medium either were doubled in concentration or were not added. The results of this experiment (table 3) indicated that all variations in this medium resulted in decreased accumulation of orthophosphate phosphorus. When the salt solution, which consisted of trace amounts of iron, manganese, magnesium and calcium, was not present phosphate accumulation did not occur and the amount of growth was negligible.

Effect of trace amounts of orthophosphate in the medium on orthophosphate accumulation during growth. Adams and Conrad reported in their studies that phosphite was not utilized by soil bacteria until all traces of phosphate in the medium were gone. Thus traces of phosphate would be scavenged by the cells for growth before phosphite oxidation could occur. Conceivably, traces of phosphate in the medium also could trigger a phosphite oxidation mechanism in these microorganisms.

The medium in which *Pseudomonas fluorescens*

strain 195 was grown in the present study contained 0.01 per cent yeast extract, which at most could provide the organisms with 44.5 μ g of phosphorus per 50 ml of medium (based on analysis provided by Difco Laboratories). The absence of yeast extract in the medium already has been shown to have a marked effect on orthophosphate accumulation (table 3).

To determine the effect of trace amounts of orthophosphate phosphorus the yeast extract of

TABLE 3

Effect of concentration of medium constituents on orthophosphate accumulation during growth of strain 195

Medium Constituents	Relative Growth; 3 Days	Orthophosphate P; 3 Days
		mg/50 ml
Control medium.....	+++	2.23
minus salt solution*.....	+	0.00
minus yeast extract.....	+++	1.37
minus iron and manganese...	+++	0.97
with 2 \times iron conc.....	+++	1.85
with 2 \times manganese conc....	+++	1.97
with 2 \times glucose conc†.....	+++	0.33
with 2 \times phosphite conc.....	+++	2.40
with 2 \times glucose and phosphite conc.....	+++	0.97

* The salt solution consisted of trace amounts of iron, manganese, magnesium, and calcium.

† The pH of this medium after 3 days of growth was 4.9.

TABLE 2

Effect of carbon source on orthophosphate accumulation during 4 days of growth of strain 195

Carbon Source	Relative Growth	Orthophosphate P in Broth Supernatants	pH
		mg/50 ml	
Glucose.....	+++	1.80	5.7
Glycerol.....	\pm	0.00	6.8
Maltose.....	\pm	0.00	7.2
Acetic acid.....	+++	0.37	9.3
Succinic acid.....	++	0.00	9.3
DL-Alanine.....	+++	0.78	9.0
DL-Alanine (minus (NH ₄) ₂ SO ₄).....	+++	0.55	9.1
Citric acid.....	++	0.00	9.2

TABLE 4

Effect of yeast extract and orthophosphate on orthophosphate accumulation during growth of strain 195

Source of Orthophosphate P	Relative Growth; 3 Days	Orthophosphate P in Supernatants	
		2 Days	3 Days
		mg/50 ml	mg/50 ml
None.....	+++	0.00	1.83
5 μ g from KH ₂ PO ₄	+++	0.00	2.02
50 μ g from KH ₂ PO ₄	+++	0.00	2.08
0.01% yeast extract.....	+++	0.87	3.07
0.01% yeast extract + 5 μ g from KH ₂ PO ₄	+++	0.75	2.93
0.01% yeast extract + 50 μ g from KH ₂ PO ₄	+++	0.75	3.03

the medium was either replaced by or added in addition to KH_2PO_4 , the latter at concentrations of 5 or 50 μg phosphorus per 50 ml of medium. Where no yeast extract was added to the medium, growth was retarded by one day and orthophosphate did not accumulate until the third day, at which time a relatively large accumulation occurred (table 4). Initial addition of trace amounts of orthophosphate to these flasks did not have any appreciable effect. Also, in media containing yeast extract the effect of orthophosphate was negligible.

DISCUSSION

It has been shown that certain microorganisms can utilize orthophosphite phosphorus as a source of phosphorus for growth. However, Robertson and Boyer (1956a) reported that organic combinations with orthophosphite were inert in biological systems. One would expect then that this anion must be oxidized to orthophosphate before utilization. Adams and Conrad (1953) reported that soil microorganisms could utilize orthophosphite for growth but that there was no accumulation of orthophosphate in the medium, although some of the phosphite phosphorus which disappeared from the medium could be recovered as orthophosphate on autolysis of the cells. Thus in some manner oxidation of orthophosphite phosphorus must have occurred within the microbial cells.

The present experiments bear out the work of Adams and Conrad but in addition show that one strain of *Pseudomonas fluorescens* (strain 195) is able not only to utilize orthophosphite for growth under aerobic conditions but also to accumulate the oxidation product, orthophosphate, in the growth medium. This accumulation occurs after most of the initial phosphorus demands for growth have been met and continues in a linear fashion as long as carbohydrate is still present. Cessation of the linear appearance of orthophosphate in the medium coincides with the onset of a maximum stationary phase of growth. Thus the accumulation of orthophosphate occurs during rapid cell multiplication and carbohydrate utilization and is not necessarily a result of death and autolysis of old cells.

The medium used in these studies would appear to be balanced especially for the oxidation of phosphite, since all variations tried in the medium resulted in decreased accumulation of orthophosphate. The mechanism by which strain 195 carries out this oxidation is presently under study.

SUMMARY

Twenty-three microbial cultures were surveyed for their ability to utilize orthophosphite phosphorus for heterotrophic growth and to accumulate the oxidation product, orthophosphate, in the growth medium. Of these 14 utilized orthophosphite for growth, but only *Pseudomonas fluorescens* strain 195 accumulated orthophosphate.

A study of orthophosphate accumulation by this pseudomonad revealed that the accumulation occurred in a linear fashion after an initial 24-hr incubation period and that the rate decreased when most of the carbohydrate had been removed from the medium. All variations tried in the growth medium resulted in reduced accumulation.

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